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Chemiluminescence of asbestos-activated macrophages

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Summary. Chemiluminescence, a measure of reactive oxygen species release by phagocytes, was compared in peritoneal exudate macrophages elicited with chrysotile asbestos, *Corynebacterium parvum* and saline. Chrysotile asbestos- and *C. parvum*-activated macrophages produced significantly more chemiluminescence than saline-elicited macrophages. In a second series of experiments the ability of opsonized chrysotile asbestos to act as a trigger for the release of chemiluminescence was tested. Opsonized chrysotile asbestos produced a dose-related release of chemiluminescence from activated macrophages except at the highest dose where chemiluminescence was reduced due, possibly, to a toxic effect of chrysotile during the assay. Opsonized latex also triggered a dose-related chemiluminescent response from activated macrophages. The potential role of toxic reactive oxygen species, released from macrophages, in the development of asbestos-related pulmonary inflammation and fibrosis are discussed.

Keywords: chemiluminescence, asbestos-activated macrophages

Pulmonary interstitial fibrosis (asbestosis) is a major disease associated with asbestos inhalation (Becklake 1976; Selikoff & Lee 1978) and is typified by increased deposition of interstitial collagen and fibroblast proliferation. Fibrosis is a common sequel to chronic inflammation and the ability of asbestos to generate inflammation in the lung has been discussed as a factor in the aetiology of asbestos-associated pulmonary interstitial fibrosis (Hamilton 1980; Miller 1978). The central role of the alveolar macrophage in interacting with inhaled particles makes it likely that alteration in the activities of these cells in the lungs of individuals inhaling asbestos could be important in the pathogenesis of asbestos-related diseases. Evidence that the macrophage may play a central role in asbestos inflammation has come from studies demonstrating macrophage-activating properties of asbestos (Davies *et al.* 1974; Hamilton *et al.* 1976; Miller & Kagan 1976;

Sirois *et al.* 1980; Donaldson *et al.* 1982, 1983*a,b*). Macrophages from inflammatory sites have been found to be activated and capable of releasing an extensive array of biologically-active substances (Allison *et al.* 1978; Nathan *et al.* 1980). One group of these secreted molecules, the reactive oxygen species (ROS): hydrogen peroxide (H_2O_2), superoxide (O_2^-), hydroxyl radical ($OH\cdot$) and singlet oxygen (1O_2), have been implicated in the tumoricidal and microbicidal functions of leucocytes (Nathan 1982) and in tissue damage at inflammatory foci (Fantone & Ward 1982).

In the present study we assessed the release of ROS from asbestos- and *Corynebacterium parvum*-elicited mouse peritoneal macrophages by measuring lucigenin-amplified chemiluminescence in response to a variety of stimuli including asbestos. Lucigenin-amplified chemiluminescence in leucocytes is a measure of superoxide release

(Allen 1981; Williams & Cole 1981b). In previous studies we have characterized the asbestos-elicited mouse peritoneal macrophage as a model asbestos-activated macrophage (Donaldson *et al.* 1982, 1983a,b).

Materials and methods

Mice. Outbred CF1 mice 6–8 weeks of age at the time of injection were used throughout.

Peritoneal exudate cells. Peritoneal exudate cells (PEC) were harvested 5 days after intraperitoneal injection of 2.5 mg chrysotile asbestos (Union International Contre Cancer sample) in 0.5 ml Dulbecco phosphate-buffered saline A (DulA), 0.5 ml DulA or 0.2 ml (0.4 mg) *Corynebacterium parvum* (heat-killed; Wellcome). Mice were killed by ether inhalation and the cells were harvested by three 2-ml washes with DulA containing 10 units/ml Heparin (Pularin) into ice-cold plastic tubes. The cells were washed, counted and maintained in Hams F₁₀ medium containing 10% heat-inactivated fetal calf serum (FCS) (GIBCO) on ice until assay when they were transferred to Hanks solution without phenol red.

Neutrophil depletion. Neutrophil depletion was by centrifugation through sodium metrizoate; 10×10^6 PEC in 5 ml of F₁₀ and 10% FCS were layered on top of sodium metrizoate (Lymphoprep; Nyygaard) in a 10-ml siliconized centrifuge tube and centrifuged at 400 *g* for 30 min. The cells at the interface were retrieved and washed before use.

Characterization of PEC. To determine the cellular composition of the PEC, cyto-spin preparations were made, stained with Wright stain and differential counts carried out.

Triggers of ROS release. The following triggers for the stimulation of ROS release were used. (a) Opsonized zymosan: zymosan was opsonized by incubation at 37°C for 30 min

in 10% Human AB serum, washed and adjusted to 10 mg/ml in Hanks solution without phenol red. (b) Opsonized latex: latex particles (Sigma, 1.09 μ m diameter) were opsonized as above and adjusted to final concentrations of 1, 5 or 10%. (c) Opsonized chrysotile asbestos: chrysotile asbestos (UICC) was opsonized as above, washed and adjusted to final concentrations of 1, 5, 10 and 20 mg/ml.

Lucigenin-amplified chemiluminescence. Chemiluminescence (CL) was measured directly in mV in an LKB 1250 luminometer. Cells were prepared at 5×10^6 /ml in cold Hanks and maintained on ice. Two hundred microlitres of cells were transferred to polystyrene cuvettes (LKB) followed by 200 μ l of lucigenin (10^{-3} M). The cuvettes were placed in the luminometer and the baseline CL was allowed to develop. When this had plateaued 500 μ l of trigger were added. Following addition of trigger, CL was monitored until it peaked.

Viability assay. To determine the extent of any toxic effect of asbestos on PEC, 50- μ l samples of cells were removed from a typical CL assay (*C. parvum* PEC; 10^{-3} M lucigenin; 10 mg/ml chrysotile) and percentage viability determined by trypan blue exclusion for 200 cells.

Use of enzymes. In order to determine the role of different reactive oxygen species in CL, superoxide dismutase (Sigma; 50 μ g/ml) and catalase (Sigma; 25 μ g/ml) were included in two separate experiments with opsonized zymosan- and *C. parvum*-elicited PEC.

Statistics. The statistical significance of replicate experiments was analysed using Student's *t*-test and analysis of variance.

Results

Characteristics of CL

Baseline CL. As discussed by Williams & Cole

(1981a) the addition of PEC and lucigenin to the polystyrene cuvettes resulted in a release of CL. Trigger was added after this reached a plateau. In each experiment this baseline CL was subtracted from the final peak CL to obtain the true peak CL due to the trigger. The magnitude of the baseline CL varied but was always low for the saline-induced PEC (0.4 ± 0.2 ; mean \pm SD) and was increased with *C. parvum*- (7.2 ± 6.5) and chrysotile asbestos- (5.4 ± 6.0) elicited PECs.

Effect of enzymes on CL. Fig. 1 shows that CL is inhibited by superoxide dismutase (SOD) and unaffected by catalase. The same result was obtained in two separate experiments.

Role of polymorphonuclear neutrophils (PMN) in CL by peritoneal exudate cells. In order to determine the role of PMN in CL by activated PEC the PEC were centrifuged through lymphoprep to reduce the proportion of granulocytes. The cells at the interface had a mean reduction in PMN of 40% but, as shown in Table 1, this had virtually no effect on peak CL when the same number of cells were compared. It was therefore assumed that PMN and macrophages from the activated PEC used in these experiments released approximately equal quantities of CL. Conse-

quently the proportion of CL due to PMN in any activated PEC was approximately equal to the percent PMN present (Table 2) and no further attempt was made to separate macrophages from neutrophils.

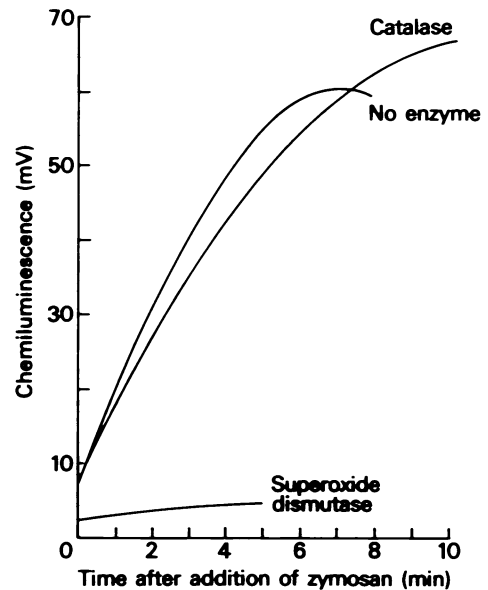


Fig. 1. Effect of superoxide dismutase (50 μ g/ml) and catalase (25 μ g/ml) on opsonized zymosan-triggered chemiluminescence of *C. parvum*-elicited PEC (500 μ l of 10 mg/ml opsonized zymosan added at time 0). The same result was obtained in two separate experiments.

Table 1. Effect of separation on sodium metrizoate on proportion of polymorphonuclear neutrophils and chemiluminescence of *C. parvum*- and chrysotile-elicited peritoneal exudate cells: opsonized zymosan as a trigger

Agent eliciting peritoneal exudate cells	Experiment no.	Polymorphonuclear neutrophils (%)		Reduction in polymorphonuclear neutrophils (%)	Change in peak chemiluminescence (%)
		Before	After		
<i>C. parvum</i>	1	23.1	12.2	47.2	-2.7
	2	18.4	8.3	54.9	-11.2
Chrysotile	1	15.7	10.7	31.8	-4.0
	2	31.8	23.3	26.7	+3.1
All experiments	—			40.6 ± 13.9	-3.1 ± 6.8

Table 2. Percent macrophages and polymorphonuclear neutrophils in various peritoneal exudate cells. Remainder of cells were lymphocytes with low (< 5%) numbers of eosinophils and basophils

Agent eliciting peritoneal exudate cells	Polymorphonuclear neutrophils (%)	Macrophages (%)	No. of experiments
Saline	5.8 ± 2.4	70.8 ± 16.2	3
<i>C. parvum</i>	15.2 ± 5.4	69.7 ± 6.2	5
Chrysotile	21.4 ± 7.3	55.8 ± 6.3	4

Results are mean ± SD.

CL responses of different PECs

Fig. 2 shows the pooled results of all experiments measuring the CL response, to opsonized zymosan, of PEC elicited with saline, *C. parvum* or chrysotile asbestos. Initial analysis of the peak CL data from all experiments (Table 3) suggested approximately equal variance in the scale of logarithm mV and analysis of variance was therefore carried out in this scale. Two effects were apparent from the analysis of variance: Firstly, small but significant systematic variation in the absolute values of peak CL between experiments. The highest and lowest values differed by a factor of 1.6 and it was not possible to identify, retrospectively, the source of this variation. Secondly, taking into account this temporal variation between experiments there were highly significant differences between the different PECs with *C. parvum* PEC being greater than chrysotile PEC by a factor of 1.3 and chrysotile PEC greater than saline PEC by a factor of 2.0.

CL in response to asbestos and latex

Fig. 3 shows a typical dose-response of CL to opsonized latex with 5-day *C. parvum*-elicited PEC. Fig. 4 shows the response of the same cells to increasing doses of opsonized chrysotile asbestos and a clear dose-response is evident over 1, 5 and 10 mg/ml; at 20

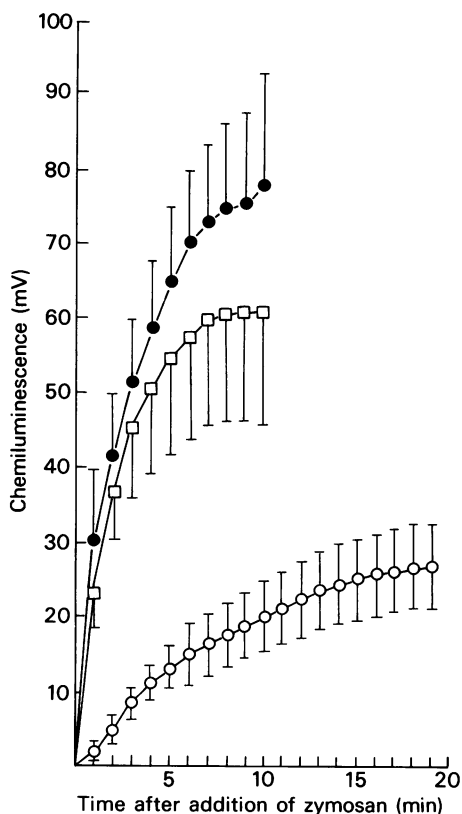


Fig. 2. Chemiluminescence response of peritoneal exudate cells elicited with saline (○), *C. parvum* (●) or chrysotile asbestos (□) to opsonized zymosan (500 µl of 10 mg/ml opsonized zymosan added at time 0). Mean ± standard deviation of three (saline), four (chrysotile) and five (*C. parvum*) separate experiments.

Table 3. Combined results of replicate experiments to measure the peak chemiluminescence response of saline-, *C. parvum*- and chrysotile-elicited peritoneal exudate cells in response to opsonized zymosan

Agent eliciting peritoneal exudate cells	Peak chemiluminescence	No. of experiments
Saline	27.4 ± 5.5	3
<i>C. parvum</i>	66.1 ± 14.2	5
Chrysotile	54.5 ± 12.7	4

Results are mean ± SD.

Analysis of variance showed significant differences between peritoneal exudate cells elicited by the three different agents: *C. parvum* > chrysotile > saline (see results).

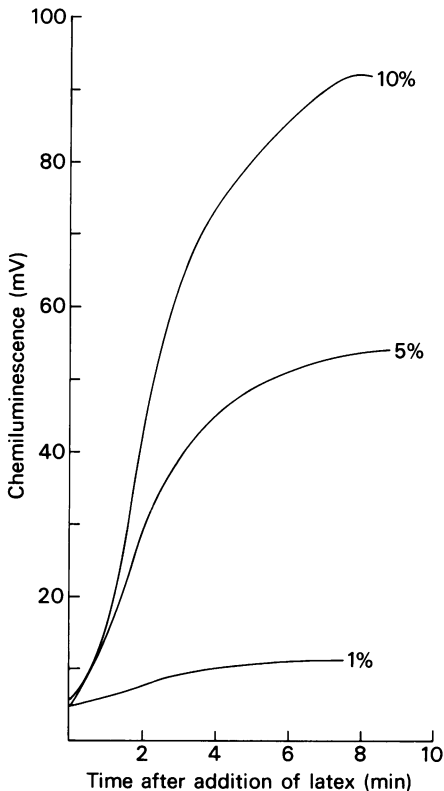


Fig. 3. Chemiluminescence response of 5-day *C. parvum* PEC to opsonized latex at 1, 5 and 10% (500 μ l of latex at the stated concentrations added at time 0). The same result was obtained in two separate experiments.

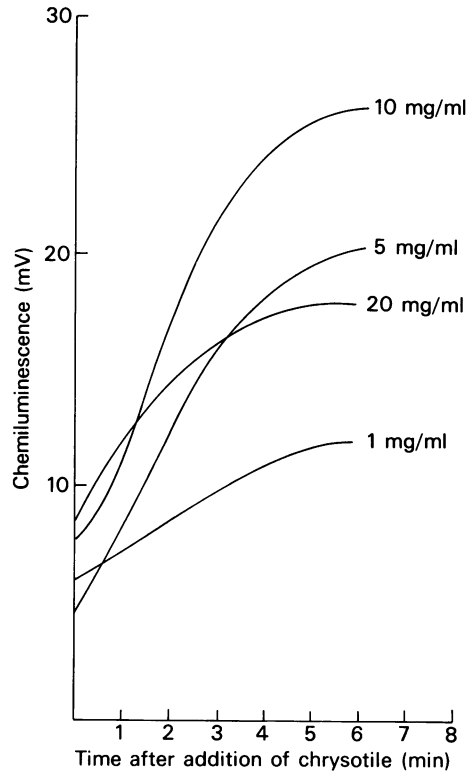


Fig. 4. Chemiluminescence response of 5-day *C. parvum*-elicited PEC to opsonized chrysotile at 1, 5, 10 and 20 mg/ml (500 μ l of opsonized chrysotile, at the stated concentrations added at time 0). The same result was obtained in two separate experiments.

mg/ml, however, the response is reduced to less than that found with 10 mg/ml.

In order to determine whether toxicity might be a factor in the loss of dose-response at high opsonized chrysotile concentration, a time course of cell viability was taken during a CL assay (*C. parvum* PEC; 10^{-3} M lucigenin; 10 mg/ml opsonized chrysotile). As shown in Fig. 5 there is 30–40% toxicity to cells within the timespan of a normal assay (10–20 min).

Table 4 shows the pooled peak CL response from replicate experiments using *C. parvum*-elicited PEC with opsonized zymosan and opsonized chrysotile as triggers. It is evident that opsonized zymosan elicited a significantly greater CL response than opsonized chrysotile on an equal-mass basis.

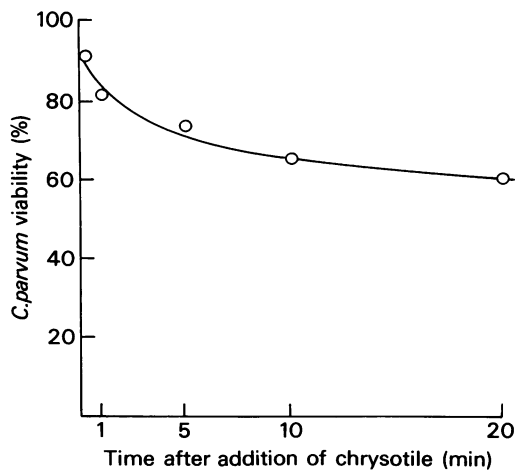


Fig. 5. Change in viability of *C. parvum* PEC during a chemiluminescence assay with 10 mg/ml opsonized chrysotile as trigger added at time 0.

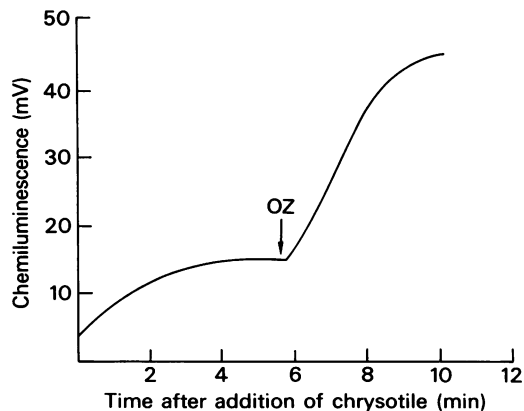


Fig. 6. Chemiluminescence response of 5-day *C. parvum* PEC to opsonized chrysotile added as 500 μ l of 10 mg/ml at time 0; on reaching a plateau opsonized zymosan was added (OZ); typical result.

Table 4. Effect of different triggers on the peak chemiluminescence response of *C. parvum* peritoneal exudate cells

Trigger	Peak chemiluminescence	No. of experiments
Opsonized zymosan	66.1 \pm 14.2*	5
Opsonized chrysotile	14.2 \pm 7.3*	5

Results are mean \pm SD.

* Significance of difference between zymosan and chrysotile: $P < 0.001$.

Figure 6 shows that on reaching a plateau of CL in response to 10 mg/ml opsonized chrysotile the cells are still sufficiently viable to respond to a further stimulus from opsonized zymosan.

Discussion

In the present study we set out to determine whether asbestos could elicit activated macrophages with increased ability to release reactive oxygen species (ROS) and also

whether asbestos could act as a trigger for the release of ROS from activated macrophages. Activated peritoneal macrophages elicited by asbestos injection were used since these have been previously characterized, while saline- and *C. parvum*-elicited macrophages were used as unactivated and activated controls respectively (Donaldson *et al.* 1982; Cullen 1978). As a measure of ROS release, lucigenin-amplified chemiluminescence (CL) was used. Chemiluminescence is correlated with other indices of the respiratory burst including O_2 consumption, hexose monophosphate shunt activity, H_2O_2 release and O_2^- release (Johnston *et al.* 1980) which are induced in phagocytes by phagocytic or membrane-perturbing stimuli (Schadelin *et al.* 1980). Lucigenin-amplified chemiluminescence is correlated with O_2^- release in macrophages (Williams & Cole 1981b) and a reaction sequence for the interaction of O_2^- with lucigenin in the generation of CL has been described (Allan 1981). In preliminary experiments we gained support for the involvement of O_2^- in CL by demonstrating the superoxide dismutase-dependent abolition of CL while catalase had no effect on CL response.

In the first set of experiments reported here the chrysotile asbestos-elicited PEC were shown to release significantly more CL than saline-elicited PEC in response to the conventional trigger of ROS release—opsonized zymosan. *C. parvum*-elicited PEC, however, released significantly more CL than chrysotile-elicited PEC and phorbol myristate acetate (PMA), a non-particulate membrane-perturbing agent, produced the same pattern of CL response from the three different PECs (data not shown). As discussed below the macrophage component of the PEC contributed the major part of the CL response.

Asbestos is thus similar to other macrophage-stimulating agents such as casein, *C. parvum*, BCG and virus infections, endotoxin and thioglycollate in causing increased chemiluminescence or ROS release in response to membrane perturbation or phagocytosis (Nathan & Root 1977; Schleupner & Glasgow 1978; Johnston *et al.* 1980).

The finding that the asbestos-elicited macrophages used here show increased CL is consistent with other markers of activation which have been reported for macrophages treated with asbestos *in vivo* and *in vitro* (Davies *et al.* 1974; Hamilton *et al.* 1976; Miller & Kagan 1976; Sirois *et al.* 1980; Donaldson *et al.* 1982, 1983a,b) and was predicted in a previous study on the basis of increased lectin-induced capping in asbestos-activated macrophages (Donaldson *et al.* 1983a).

From the outset of these studies we were primarily interested in the macrophage component of the PEC although substantial numbers of PMNs could be present in some preparations. Depletion of PMNs from within activated PEC samples, by centrifugation through lymphoprep, resulted in an average reduction in PMNs of 40% with no appreciable reduction in the CL response of these PEC. We therefore concluded that CL of activated macrophages and PMNs was equal in magnitude on a cell-for-cell basis. Consequently the CL due to PMNs in any activated PEC was equivalent to the

percentage of PMN present in the PEC. It is important to note, however, that the study of Sykes *et al.* (1982), and our own unpublished data, indicate that pulmonary deposition of pathogenic dust may well result in the presence of PMNs amongst the free alveolar cell population where they could contribute significantly to the accumulation of ROS if appropriately stimulated. Mixed macrophage/PMN preparations therefore constitute a relevant population for study.

The second set of experiments described above confirm that asbestos can act as a trigger for the release of ROS from activated macrophages, as measured by CL, although the response to opsonized chrysotile was much less than that produced by opsonized latex. The dose dependency of the CL response to chrysotile asbestos which was evident at the low and intermediate doses was lost at the highest dose (20 mg/ml) due possibly to a toxic effect of such a large asbestos dose; testing for cell viability during an assay confirmed a toxic effect of asbestos under the conditions of the assay. This toxicity was evident despite the fact that the asbestos fibres were opsonized with human serum and the acute toxic effects of asbestos are reduced by protein coating of the active fibre surface (Miller 1978). However, increased susceptibility of activated macrophages to the toxic effects of asbestos *in vitro*, in the presence of serum, has been previously reported from our laboratory (Wright *et al.* 1983).

We appreciate that the asbestos dose used to trigger CL in the present study was very high and far in excess of those encountered, for example, in the lungs of animals inhaling asbestos. The relatively low sensitivity of the chemiluminescence amplification and detection system, however, necessitated the use of such high doses and we do not believe that this diminishes the potential relevance of the findings.

Two recent studies have reported the effects of asbestos in triggering CL responses in phagocytes; these studies revealed that

peripheral blood PMNs could be stimulated by asbestos to release ROS (Doll *et al.* 1982a) while peripheral blood monocytes did not show this response (Doll *et al.* 1982b). Our use of activated macrophages as indicator cells in the present study is supported by reports of the macrophage-activating potential of asbestos (Davies *et al.* 1974; Hamilton *et al.* 1976; Miller & Kagan 1976; Sirois *et al.* 1980; Donaldson *et al.* 1982) and the central role of activated macrophages in inflammation (Allison *et al.* 1978).

The in-vivo relevance of the present findings lies in the possibility that activation of macrophages in the lungs of individuals inhaling asbestos could lead to localized accumulation of ROS. We do, however, recognize the metabolic and functional differences between peritoneal and alveolar macrophages although ROS release by alveolar macrophages has been reported (Williams & Cole 1981b). In this regard it is notable that Miller & Kagan (1976) have reported evidence of alveolar macrophage activation in the lungs of rats inhaling crocidolite asbestos.

Evidence that ROS could have local toxic and tissue damaging effects is derived from experiments where both cell-free enzyme system-derived, and phagocyte-derived, oxygen radicals have been found to cause endothelial cell damage (Sacks *et al.* 1978), red cell lysis (Kellogg & Fridovich 1977), fibroblast toxicity (Simon *et al.* 1981) and autotoxicity to PMNs (McCord & Wong 1979). These toxic effects are thought to be brought about largely by peroxidation of membrane lipids leading to structural disorganization of the cell membrane (Slater 1979). It is possible therefore that, in the lungs of individuals inhaling asbestos, a factor in the maintenance of an inflammatory response is localized release of increased levels of ROS. The asbestos-activated macrophages could be envisaged as being primed to produce increased amounts of ROS, the release of which could be triggered by contact with any of a number of inhalable particles reported to have this triggering

property such as bacteria, yeast (Williams *et al.* 1980), pollen (Lindberg *et al.* 1982) and, as shown in the present study, asbestos itself. If an excess of ROS in the alveolar spaces overcame the normal scavenging systems this could lead to epithelial damage with subsequent activation of inflammatory cascades leading ultimately to fibrosis (Pickrell 1981).

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